The riboflavin transporter RibU in Lactococcus lactis

Burgess, CM; Slotboom, DJ; Geertsma, ER; Duurkens, Hinderika; Poolman, B; van Sinderen, D

Published in:
Journal of Bacteriology

DOI:
10.1128/JB.188.8.2752-2760.2006

JOURNAL OF BACTERIOLOGY, Apr. 2006, p. 2752–2760 Vol. 188, No. 8
0021-9193/06/$08.00+0 doi:10.1128/JB.188.8.2752–2760.2006

The Riboflavin Transporter RibU in *Lactococcus lactis*: Molecular Characterization of Gene Expression and the Transport Mechanism

Catherine M. Burgess,[1] Dirk Jan Slotboom,[2] Eric R. Geertsma,[2] Ria H. Duurkens,[2] Bert Poolman,[2] and Douwe van Sinderen[1,3]*

Department of Microbiology and Biosciences Institute, National University of Ireland Cork, Western Road, Cork, Ireland[1]; Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands[2]; and Alimentary Pharmabiotic Centre, Biosciences Institute, National University of Ireland Cork, Western Road, Cork, Ireland[3]

Received 12 October 2005/Accepted 6 February 2006

This study describes the characterization of the riboflavin transport protein RibU in the lactic acid bacterium *Lactococcus lactis* subsp. *cremoris* NZ9000. RibU is predicted to contain five membrane-spanning segments and is a member of a novel transport protein family, not described in the Transport Classification Database. Transcriptional analysis revealed that *ribU* transcription is downregulated in response to riboflavin and flavin mononucleotide (FMN), presumably by means of the structurally conserved RFN (riboflavin) element located between the transcription start site and the start codon. An *L. lactis* strain carrying a mutated *ribU* gene exhibits altered transcriptional control of the riboflavin biosynthesis operon *ribGBAH* in response to riboflavin and FMN and does not consume riboflavin from its growth medium. Furthermore, it was shown that radiolabeled riboflavin is not taken up by the *ribU* mutant strain, in contrast to the wild-type strain, directly demonstrating the involvement of RibU in riboflavin uptake. FMN and the toxic riboflavin analogue roseoflavin were shown to inhibit riboflavin uptake and are likely to be RibU substrates. FMN transport by RibU is consistent with the observed transcriptional regulation of the *ribGBAH* operon by external FMN. The presented transport data are consistent with a uniport mechanism for riboflavin translocation and provide the first detailed molecular and functional analysis of a bacterial protein involved in riboflavin transport.

The water-soluble vitamin riboflavin (vitamin B_2) is the precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide. These flavins are essential cofactors in enzymes catalyzing redox reactions and are obtained by phosphorylation of riboflavin in all living organisms. Plants, fungi, and many microorganisms have the biosynthetic ability to synthesize riboflavin, whereas higher animals must obtain it from their diet. The abilities of various gram-positive and gram-negative bacteria to synthesize riboflavin de novo have been described by various authors (3, 10, 27).

Certain bacteria such as *Escherichia coli* are dependent on endogenous biosynthesis because they apparently lack an uptake system for the vitamin (2). It is for this reason that *E. coli* riboflavin auxotrophs require extremely high levels of riboflavin (720 μM) in their growth medium, and it is likely that at this concentration the vitamin crosses the membrane by diffusion rather than by means of a dedicated transport system. *Bacillus subtilis* not only possesses a functional riboflavin biosynthetic pathway but can also import the vitamin from the growth environment (6). The *ypaA* gene of *Bacillus subtilis* has been suggested to be a riboflavin transporter (17), since a *B. subtilis* riboflavin auxotroph carrying a mutated *ypaA* gene was shown to require unusually high riboflavin concentrations in its growth medium. Although the role of the *ypaA* gene in riboflavin transport has not been unequivocally demonstrated, its putative function is consistent with the presence, immediately upstream of the *ypaA* coding sequence, of a so-called RFN element (11). RFN elements are found upstream of prokaryotic riboflavin biosynthesis operons, where they play a regulatory role in the expression of the riboflavin biosynthesis genes by a mechanism involving FMN-mediated folding of the RFN-encompassing mRNA that causes premature transcription termination and/or ribosome seclusion (25, 41).

In *Lactococcus lactis*, the riboflavin biosynthesis operon has recently been characterized and the RFN element was shown to be instrumental in transcriptional regulation of the riboflavin biosynthesis genes (4). An *L. lactis* strain containing a mutation in the *ribA* gene, which is essential for riboflavin biosynthesis and codes for a GTP cyclohydrolase II/3,4-dihydroxy-2-butanone-4-phosphate synthase, is dependent on the presence of riboflavin in the growth medium (4). However, such a mutant needed much lower levels of riboflavin (5 μM) than the levels required by *E. coli* riboflavin auxotrophs (720 μM), suggesting the presence of a dedicated transport system in *L. lactis*. The current study describes the characterization of a homologue of *ypaA*, designated *ribU*, in *Lactococcus lactis* subsp. *cremoris* NZ9000 and demonstrates its direct role in riboflavin uptake.

MATERIALS AND METHODS

Bacterial strains and plasmids, media, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *L. lactis* strains were grown in M17 medium supplemented with 0.5% glucose (GM17) (38) or in chemically defined medium (CDM) (adapted by removal of folic acid, riboflavin, and nucleotides) (26, 28). Where appropriate, growth medium contained tetracycline (5 μg ml^{-1}) or X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg ml^{-1}).

* Corresponding author. Mailing address: Alimentary Pharmabiotic Centre, Department of Microbiology and Biosciences Institute, National University of Ireland Cork, Western Road, Cork, Ireland. Phone: 353214901365. Fax: 353214903101. E-mail: d.vansinderen@ucc.ie.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
NZ9000	MG1363 *pepN*::*nisRK*, wild-type strain	19
CB010	Riboflavin-overproducing spontaneous mutant of NZ9000	4
NZ9000Δ*ribA*	NZ9000 derivative with a 783-bp deletion in *ribA*	4
NZ9000Δ*ribU*	NZ9000 derivative with a 414-bp deletion encompassing P_{ribU} and 69 bp of *ribU*	This study
Plasmids		
pORI280	Em^r $LacZ^+$ ori^+ of pWV01, replicates only in strains where *repA* is provided in *trans*	21
pORI280Δ*ribU*	pORI280 derivative containing a truncated version of NZ9000 *ribU* and the surrounding regions	This study
pPTPL	Tet^r $LacZ^+$; promoter probe vector	4
pPTPL*ribU*	pPTPL derivative containing the promoter region of NZ9000 *ribU*	This study
pPTPLop	pPTPL derivative containing the promoter region of NZ9000 *rib* operon	4

Bioinformatics. All sequence data were obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Sequence database interrogations were performed using BLASTP (1). Potential membrane-spanning regions were identified using DAS (dense alignment surface) (7), TMHMM (transmembrane hidden Markov model) (18), and TmPRED (transmembrane prediction) (13). The proteins were also analyzed for similarity to families of transport proteins using the BLAST program on the Transport Classification Database (www.tcdb.org) (5). Potential RFN elements were identified using RFAM (RNA family) (http://www.sanger.ac.uk/Software/Rfam/) (12). Folding of the 5′ mRNA leader region was predicted using mFOLD (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/) (43).

DNA manipulations and transformations. Plasmid DNA was isolated from *E. coli* using the JETquick plasmid miniprep kit (Genomed, Löhne, Germany), following the instructions of the manufacturer. Plasmid DNA was isolated from *L. lactis* using the same kit except that cells were preincubated in cell resuspension solution containing 20 mg ml^{-1} lysozyme at 55°C for 30 minutes to effect cell lysis. Transformation of *E. coli* was carried out as described by Sambrook and Russell (36). Transformation of *L. lactis* was achieved according to the protocol of de Vos et al. (9). Chromosomal DNA was isolated from *L. lactis* as described previously (22, 23). Southern blot assays were carried out using a standard protocol (36), and detection was accomplished using ECL labeling (Amersham, Buckinghamshire, United Kingdom) according to the manufacturer's instructions.

Plasmid construction. Primers containing a BglII site and an XbaI recognition sequence within the forward and reverse primer, respectively, were used to amplify the predicted promoter and regulatory region of *ribU*. The amplified product was digested with BglII and XbaI and cloned into the promoter probe vector, pPTPL (4), digested with the same two enzymes. The resulting plasmid, listed in Table 1, was constructed using *E. coli* EC1000 as a cloning host and was subsequently transferred to the lactococcal strain NZ9000. X-Gal was used in plates as a qualitative indicator of promoter activity.

Transcriptional analysis. β-Galactosidase assays (16) were performed on crude cell extracts of *L. lactis* strains which had been grown in CDM without riboflavin or CDM supplemented with 5 μM or 50 μM riboflavin or 5 μM FMN as appropriate. Total RNA was isolated at mid-logarithmic phase by the Macaloid method (20) from *L. lactis* strains grown in CDM in the presence or absence of 5 μM riboflavin. Northern hybridization analysis was performed by denaturing 5 μg RNA at 65°C and followed by electrophoretic separation on a 0.8% formaldehyde agarose gel. RNA was then transferred to a Hybond N^+ charged nylon membrane (Amersham, Buckinghamshire, United Kingdom) by capillary transfer using 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as transfer buffer. Purified *ribU*-encompassing PCR product was used as a probe and was labeled with [α-^{32}P]dATP with the Prime-a-Gene kit (Promega, Madison, Wis.) according to the manufacturer's instructions. The prehybridization and hybridization steps were carried out at 48°C in 10 ml UltraHyb (Ambion, Austin, Tex.), and washes were executed at 48°C according to the manufacturer's instructions. Detection was performed by exposure to a Kodak Biomax MR film at −70°C for 4 h.

Determination of transcription start site. A reverse primer was designed approximately 120 bp downstream of the assumed transcription start site upstream of *ribU*. Primer extension analysis was performed by annealing 10 pmol of 5′-γ-^{32}P-labeled primer to 50 μg NZ9000 RNA isolated from mid-logarithmic-phase cells (32). A GATC sequence ladder which was run alongside the primer extension product was produced using the same labeled primer with the T7 DNA polymerase sequencing kit (USB Corp., Ohio). Detection was carried out by exposure to Kodak Biomax MR film at −70°C for 48 h.

Construction of a chromosomal deletion in *ribU*. Primers were designed to amplify the sections overlapping and flanking either end of *ribU* and its promoter region. Splicing by overlap extension PCR (15) was used to create a PCR product which contained a 414-bp deletion encompassing the entire promoter and regulatory region and the first 23 codons of *ribU*. This PCR product was inserted into pORI280 (Table 1) using the NcoI and BamHI restriction sites present on the outermost primers. The resulting plasmid, designated pORI280Δ*ribU*, was used to introduce the deletion into the NZ9000 chromosome by replacement recombination (21), creating strain NZ9000Δ*ribU*. PCR, sequencing, and Southern blot analyses were used to confirm the integrity of the deletion.

Quantitative analysis of riboflavin in culture medium. Extracellular riboflavin concentrations were measured using reverse-phase high-pressure liquid chromatography (HPLC) as described previously (4). Samples taken from GM17 were diluted 1 in 2 with HPLC-grade water before analysis. Commercially obtained riboflavin was used as a reference and to obtain a standard curve.

Riboflavin uptake using whole cells. Cultures were grown to early stationary phase in GM17 (optical density at 600 nm [OD_{600}] of approximately 1.8), and cells were then harvested by centrifugation at 7,700 × *g* at 4°C for 10 min. Harvested cells were washed with 50 mM KP_i, pH 7.0; resuspended in the same buffer to obtain a cell suspension with an OD_{600} of 10; and kept on ice until use. Before each uptake assay, the cells were preenergized for 5 min with 10 mM glucose at 30°C or deenergized with 10 mM 2-deoxyglucose (30, 39).

Uptake was started by the addition of [^{3}H]riboflavin (Campro Scientific, Veenendaal, The Netherlands) to a final concentration of 1 μM, unless otherwise specified. The sample volume was 200 μl, and at the indicated time points uptake was quenched with ice-cold 50 mM KP_i, pH 7.0, and rapidly filtered through 0.45-μm cellulose nitrate filters (Schleicher and Schuell GmbH, Dassel, Germany). The filters were washed once with 2 ml ice-cold 50 mM KP_i, pH 7.0, and then dried using an infrared lamp, and the radioactivity was determined by liquid scintillation counting. In some cases roseoflavin or FMN was added at various concentrations at the same time as the radiolabeled riboflavin.

For imposition of an artificial proton motive force, NZ9000 cells were washed in 50 mM KP_i, pH 7.0, plus 100 mM potassium acetate; treated for 10 min at 30°C with 10 mM 2-deoxyglucose (Fluka, Buchs, SG, Switzerland) to deenergize the cells; and then resuspended in 50 mM KP_i, pH 7.0, plus 100 mM potassium acetate and 10 mM 2-deoxyglucose to a final OD_{600} of 100. The cells were then diluted 1 to 10 in either 50 mM KP_i, pH 7.0, plus 100 mM potassium acetate (no gradient) or 50 mM NaP_i, pH 7.0, plus 100 mM NaCl in order to generate a proton motive force. In each case, the outside buffer contained valinomycin at a final concentration of 2 μM. Dilution of the energy-depleted cells in the sodium ion-containing buffer will create a membrane potential (by valinomycin mediated K^+ efflux down the concentration gradient), a pH gradient (by efflux of acetic acid directly through the lipid bilayer), and a Na^+ ion gradient (imposed by the buffer composition). For an overview of the procedures see the work of Poolman et al. (29). Either [^{3}H]riboflavin at a final concentration of 1 μM or [^{14}C]alanine at a final concentration of 1.7 μM was used. At various time points, the reaction mixtures were quenched with ice-cold outside buffer and the cells were rapidly filtered through cellulose nitrate filters. For the calculations, it was assumed that an *L. lactis* cell suspension with an OD_{660} of 5 contains 1 mg of protein per ml (31).

Nucleotide sequence accession numbers. The nucleotide sequence data for *L. lactis* subsp. *cremoris* NZ9000 *ribU* and the regulatory region reported in this

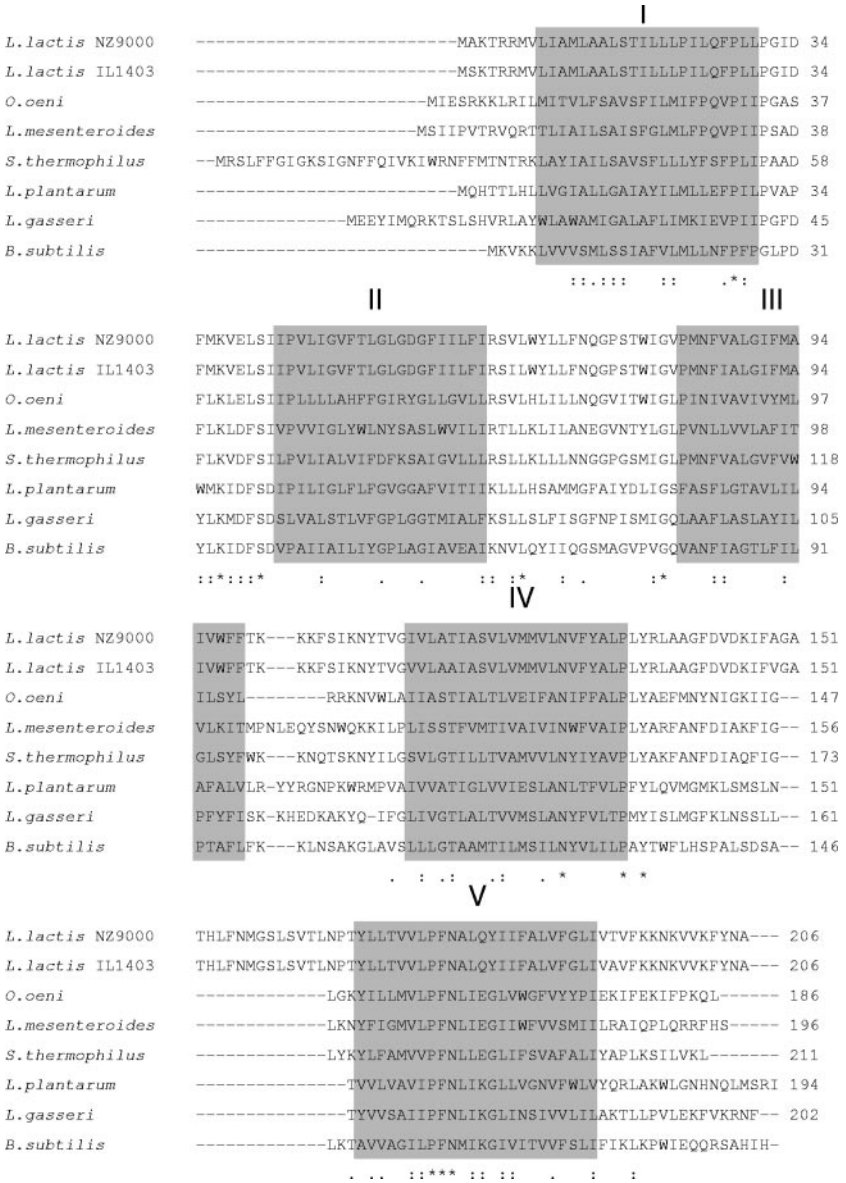

FIG. 1. Alignment of RibU homologues from various bacterial strains. The positions of the predicted TMSs are shaded and numbered I to V. The region between TMSs IV and V is predicted to be membrane spanning in both *L. lactis* RibU proteins, while in the other aligned sequences a large part of this region is absent and not predicted to be traversing the membrane.

paper have been submitted to the GenBank database under accession number AY994156.

RESULTS

Identification of a putative riboflavin transporter, RibU, in *L. lactis* NZ9000. The amino acid sequence of a putative riboflavin transporter, encoded by *ypaA* in *B. subtilis* (17), was employed to identify a single 618-bp homologue, designated here as *ribU*, on the *L. lactis* MG1363 genome. In a situation analogous to that in *B. subtilis* (41), a conserved RNA regulatory region is present upstream of *ribU*. Homologues of RibU and YpaA are present in gram-positive bacteria belonging to the *Bacillales*, the *Lactobacillales*, and the clostridia as well as in *Thermotoga maritima* and in archaea belonging to the *Thermococcales* (*Pyrococcus* and *Thermococcus* species). These proteins have a length of approximately 200 amino acids. A multiple sequence alignment of selected RibU homologues is shown in Fig. 1.

RibU and its homologues were analyzed for the presence of transmembrane segments (TMSs) using a number of computational tools. Although RibU from *L. lactis* is predicted to contain six TMSs by TMHMM and DAS (7, 18) (Fig. 1), the region corresponding to the fifth TMS is lacking in the other identified homologous proteins including YpaA (represented by gaps in the multiple sequence alignment). Since the overall structure and topology of these highly similar proteins are most likely conserved, the predicted fifth TMS in RibU may actually be part of a loop structure. Figure 1 shows an alignment of the

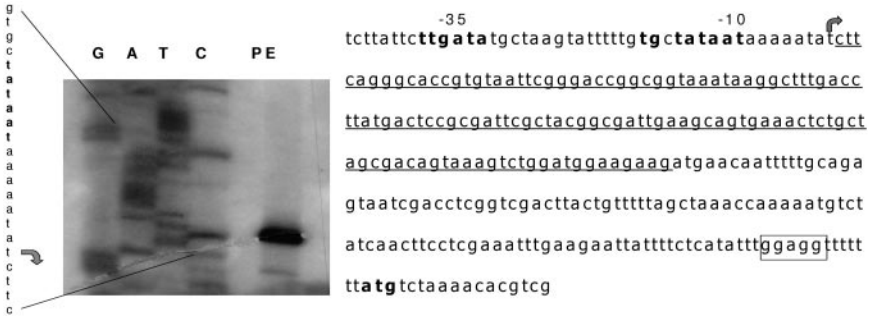

FIG. 2. Primer extension (PE) analysis of P_{ribU} run alongside a sequencing ladder. The deduced −35 and −10 boxes are indicated by boldface in the sequence displayed on the right side of this figure. The bent arrow indicates the identified transcription start site. The identified RFN element is underlined. The assumed ribosomal binding site is boxed, and the *ribU* start codon is in boldface.

proteins with the TMSs predicted by TMHMM highlighted. Neither RibU nor any of the homologues displayed significant sequence similarity to any known family of the Transport Classification Database (5) (data not shown).

The DNA regions upstream of the *ribU* homologues were analyzed for the presence of a potential RFN element using RFAM (12). All identified *ribU* genes are predicted to possess an RFN element in the region upstream of *ribU*. These upstream regions were analyzed using the program mFOLD, which predicts RNA and DNA folding structures (43). For *ypaA* of *B. subtilis*, it has been suggested that regulation occurs at the level of translation by means of a Shine-Dalgarno sequence sequestering mechanism (41). For a number of the putative regulatory elements analyzed in this study a similar Shine-Dalgarno sequence sequestering mechanism may be predicted, as such folding is energetically favorable. However, in NZ9000 a putative rho-independent terminator structure can be identified in this conserved regulatory region, which suggests that regulation takes place at the level of transcription. Such a terminator structure is also present in the leader region of *Lactobacillus plantarum* WCFS1 (data not shown).

Transcriptional analysis of *ribU* in NZ9000 and the riboflavin-overproducing derivative CB010. In order to identify the promoter, P_{ribU}, of the *L. lactis* NZ9000 *ribU* gene, primer extension analysis was performed (Fig. 2). The transcription start site was identified as a thymine, upstream of which −10 and −35 sequences were identified with a clear resemblance to the consensus vegetative RNA polymerase recognition sequences for *L. lactis* (8). To analyze transcription of *ribU* in further detail, Northern hybridization was carried out using a PCR product encompassing *ribU* as a probe with RNA isolated from *L. lactis* strains NZ9000 and CB010, grown to mid-logarithmic phase in the presence or absence of 5 μM riboflavin. *L. lactis* CB010 is a roseoflavin-resistant mutant that constitutively produces riboflavin due to deregulation of transcription of the *rib* operon (4). This analysis showed that *ribU* is transcribed as a monocistronic transcript with an estimated length of 0.95 kb (Fig. 3A). This transcript would encompass the predicted RFN element and is predicted to end at a putative rho-independent terminator located downstream of *ribU*. The highest level of *ribU* transcription occurred when NZ9000 was grown in the absence of riboflavin, although a low level of transcription was still observed in the presence of the vitamin (lanes 1 and 2, Fig. 3A). In contrast, for CB010 *ribU* transcription was undetectable regardless of the exogenous riboflavin status, indicating that RibU is not essential for riboflavin pro-

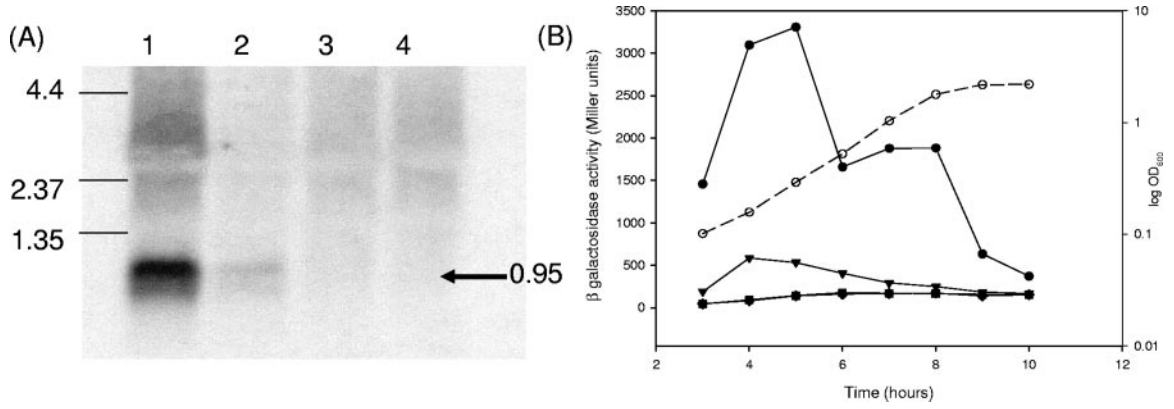

FIG. 3. Transcriptional analysis of *ribU*. (A) Northern hybridization analysis of *ribU* in NZ9000 and CB010. Lane 1, NZ9000 RNA from CDM; lane 2, NZ9000 RNA from CDM plus 5 μM riboflavin; lane 3, CB010 RNA from CDM; lane 4, CB010 RNA from CDM plus 5 μM riboflavin. An RNA size ladder (in kilobases) is indicated on the left. The size of the transcripts is indicated to the right. (B) β-Galactosidase activities of NZ9000 containing pPTPL*ribU* grown in CDM or CDM plus 5 μM riboflavin are represented by circles and inverted triangles, respectively. β-Galactosidase activities produced by CB010 containing pPTPL*ribU* grown in CDM or CDM plus 5 μM riboflavin are represented by squares and diamonds, respectively. The dashed line indicates growth of the strains plotted on a semilog scale.

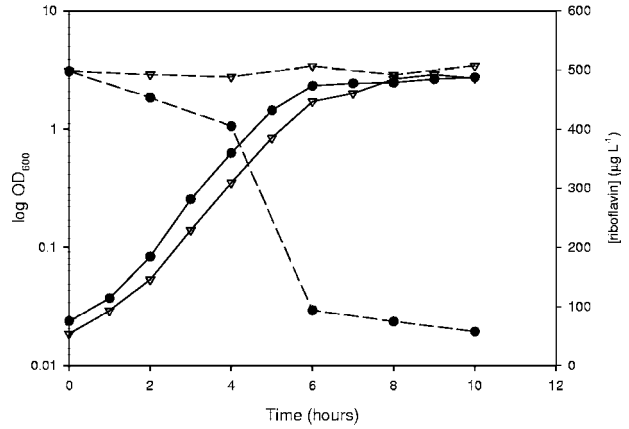

FIG. 4. Analysis of growth and riboflavin levels of NZ9000 and NZ9000Δ*ribU* in GM17. The solid lines represent log OD_{600}, and the dashed lines represent riboflavin levels as measured by HPLC in the cell-free supernatant following growth. Data obtained using NZ9000 are shown with black solid circles, and those obtained using NZ9000Δ*ribU* are depicted with empty inverted triangles.

duction. This apparent transcriptional regulation is consistent with the results from the analysis of NZ9000 and CB010 containing a P_{ribU}-*lacZ* transcriptional fusion on plasmid pPTPL *ribU* (Fig. 3B), which showed that in the absence of riboflavin P_{ribU} activity is high in NZ9000, while in the presence of the vitamin in the growth medium the activity of this promoter is low. In contrast, when the *ribU*-reporter fusion plasmid was present in CB010, essentially no β-galactosidase activity was observed, regardless of the presence of extracellular riboflavin.

Chromosomal deletion of *ribU* in NZ9000. It has previously been shown that NZ9000 consumes riboflavin from its growth medium, if the vitamin is present (37). To examine whether *ribU* in *L. lactis* plays a role in this observed vitamin consumption phenotype, a deletion strain, designated NZ9000Δ*ribU*, was created in which the *ribU* promoter, the presumed regulatory region, translation initiation signals, and the first 23 codons of *ribU* were removed from the genome. To determine whether this partial *ribU* deletion affects riboflavin consumption, strains NZ9000 and NZ9000Δ*ribU* were grown in GM17. Samples were taken from the cultures periodically, and the riboflavin content of the cell-free supernatant was determined. Figure 4 illustrates that, in contrast to the wild-type strain, NZ9000Δ*ribU* does not consume riboflavin from the medium, indicating that the riboflavin uptake mechanism has been rendered nonfunctional. The riboflavin-consuming phenotype could be restored in NZ9000Δ*ribU* by complementation in *trans*, that is, following introduction of intact *ribU* on a plasmid (data not shown).

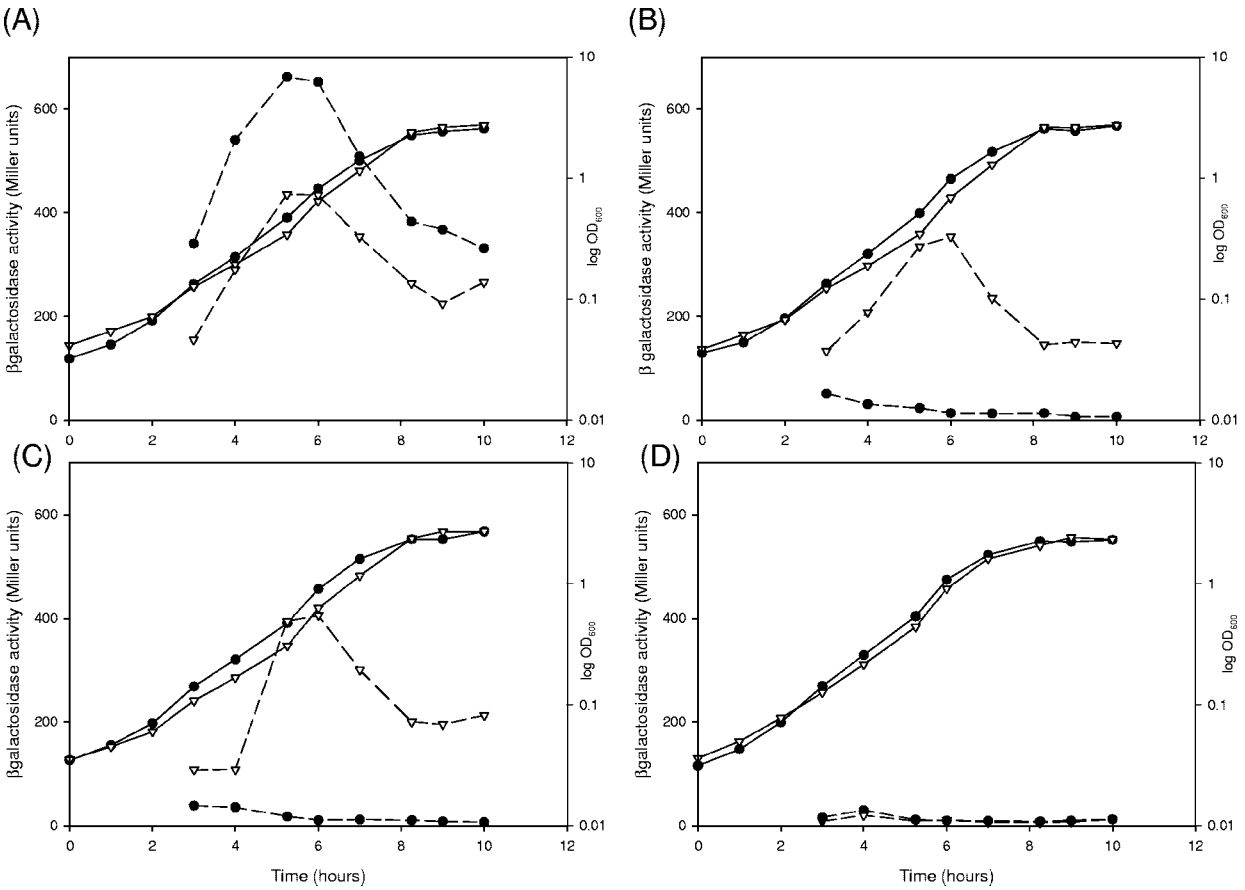

FIG. 5. $P_{ribGBAH}$ activity in NZ9000 and NZ9000Δ*ribU* in various media. The solid circles represent NZ9000, and the empty inverted triangles represent NZ9000Δ*ribU*. The solid lines represent growth (on a semilog scale), and the dashed lines represent β-galactosidase activity. (A) CDM; (B) CDM plus 5 μM riboflavin; (C) CDM plus 5 μM FMN; (D) CDM plus 50 μM riboflavin.

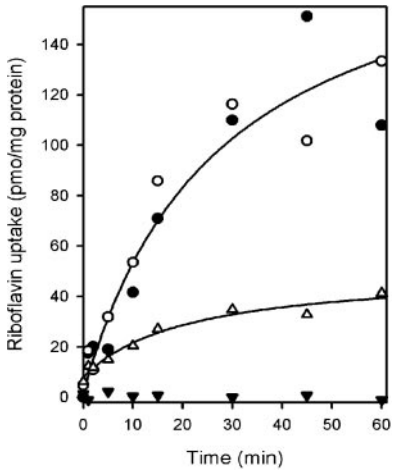

FIG. 6. Uptake of [^{3}H]riboflavin in whole cells. Cells concentrated to an OD_{600} of 10 were energized for 5 min with glucose, and uptake was started by the addition of 1 μM [^{3}H]riboflavin. At given time points, the uptake was stopped with ice-cold buffer, the samples were filtered, and radioactivity was counted. Black circles represent NZ9000, open circles represent NZ9000Δ*ribA*, and black inverted triangles represent NZ9000Δ*ribU*. Open triangles represent NZ9000 cells that were deenergized with 2-deoxyglucose.

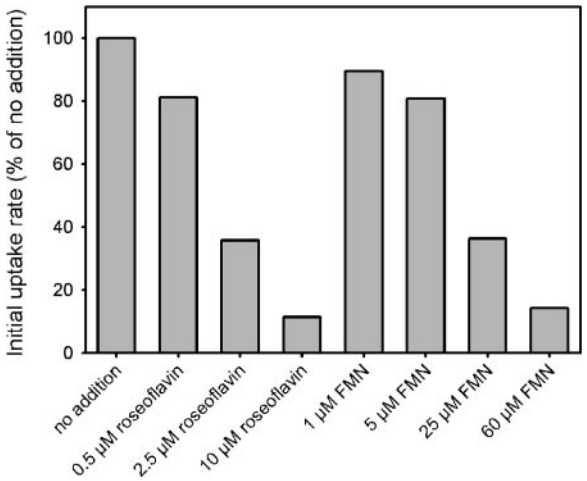

FIG. 7. Competition of riboflavin uptake in whole cells (NZ9000) by FMN and roseoflavin. For the uptake assays, no additions, 0.5 to 10 μM roseoflavin, or 1 to 60 μM FMN was supplemented concomitantly with [^{3}H]riboflavin. The concentration of [^{3}H]riboflavin in each experiment was 1 μM. Initial uptake rates were determined from the riboflavin uptake after 4 min. One hundred percent corresponds to a riboflavin uptake rate of 5 pmol/mg of protein/min.

Effect of chromosomal deletion of *ribU* on transcription of the riboflavin biosynthesis operon. To study the effect of the *ribU* deletion on expression of the riboflavin biosynthesis genes, the activity of $P_{ribGBAH}$ was examined in the wild-type strain NZ9000 and in NZ9000Δ*ribU*, grown in CDM or CDM supplemented with 5 or 50 μM riboflavin or 5 μM FMN. In CDM high promoter activity of $P_{ribGBAH}$ was observed in both NZ9000 and NZ9000Δ*ribU*, although for unknown reasons it is lower in the latter strain (Fig. 5A). Regardless of the presence of 5 μM riboflavin or 5 μM FMN the activity of the $P_{ribGBAH}$-*lacZ* fusion in NZ9000Δ*ribU* was high (Fig. 5B and C). This is in contrast to the wild-type situation where $P_{ribGBAH}$ activity was dramatically reduced in the presence of this concentration of riboflavin or FMN. However, when the riboflavin concentration in the medium was increased from 5 to 50 μM, the $P_{ribGBAH}$ activity was reduced in both NZ9000 and NZ9000Δ*ribU* (Fig. 5D). This suggests that, in the presence of high levels of riboflavin, the vitamin is able to enter the cell independently of RibU and consequently exert its effects on transcription of the *rib* operon.

Riboflavin uptake and substrate specificity of RibU. Riboflavin transport was analyzed in glucose-metabolizing whole cells of NZ9000, NZ9000Δ*ribU*, and NZ9000Δ*ribA*, a strain rendered incapable of riboflavin biosynthesis (4). Both the wild-type strain NZ9000 and NZ9000Δ*ribA* showed high rates of riboflavin transport (Fig. 6) and reached similar end levels of uptake. The level of accumulation of riboflavin was approximately 30-fold, assuming a specific internal volume of 3 μl per mg of protein. In agreement with the lack of riboflavin consumption from the external medium, essentially no uptake of riboflavin could be measured in NZ9000Δ*ribU*. In deenergized wild-type cells (depleted of ATP and the proton motive force [Δ*p*] poised to zero by incubation with 2-deoxyglucose) the uptake of riboflavin was significantly lower than in glucose-metabolizing cells but still an apparent eightfold accumulation was found.

To determine whether FMN and roseoflavin are substrates of RibU, these compounds were used in competition assays (Fig. 7). Clearly, both roseoflavin and FMN inhibited uptake of radiolabeled riboflavin, but higher concentrations of FMN than roseoflavin were needed to compete with riboflavin uptake. Although the inhibition of riboflavin uptake by FMN and roseoflavin could be due to binding of these compounds to RibU without transport taking place, transport of FMN by RibU would be consistent with the reduction of the activity of the $P_{ribGBAH}$ promoter by FMN in the growth medium that is observed in the wild-type strain but not in the RibU deletant (Fig. 5C).

To determine the driving force for riboflavin accumulation in the wild-type strain an artificial Δ*p* was generated in cells that previously had been deenergized (depleted of ATP and Δ*p* poised to zero). The proton motive force was not capable of driving accumulation of riboflavin (Fig. 8A). The amount of riboflavin taken up in cells with an artificially imposed Δ*p* was similar to the uptake observed in deenergized cells. As a control for the generation of the proton motive force, uptake of radiolabeled L-alanine was measured and was found to be significantly higher in cells containing an artificially imposed Δ*p* than in deenergized cells (Fig. 8A, inset).

The apparent accumulation of radiolabeled riboflavin, which is not dependent on the proton motive force, is likely to be driven by equilibration of internal and external riboflavin pools via an exchange (counterflow) mechanism. This possibility is supported by the fact that accumulated radiolabeled riboflavin in energized cells could be chased out of the cell with excess nonlabeled external riboflavin (Fig. 8B). Exchange would also explain the low but significant apparent riboflavin uptake in deenergized cells, because deenergized cells may not be completely depleted of riboflavin. Because the RibU primary sequence does not reveal any indications for a role of ATP (no Walker A and B motifs, no similarity to ABC or P-type

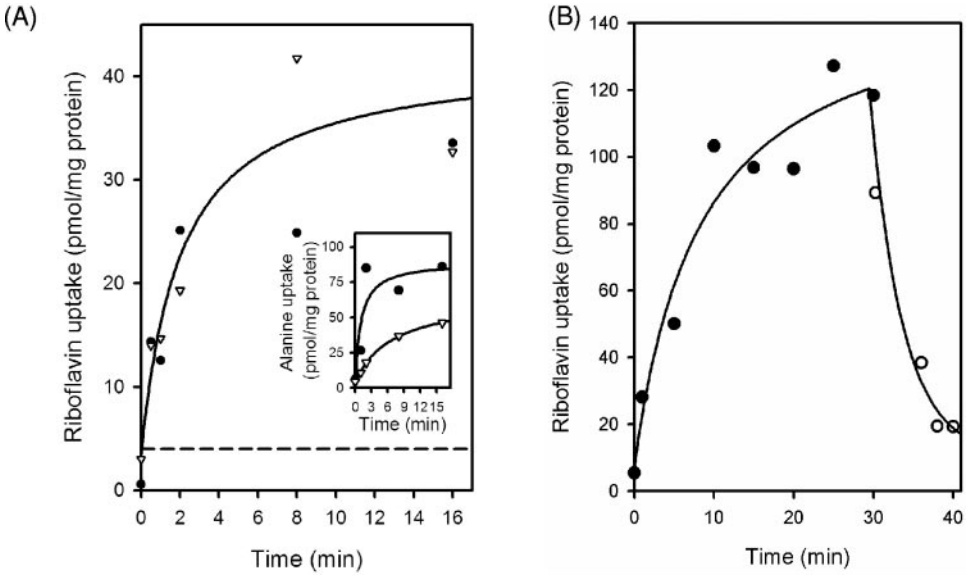

FIG. 8. (A) Effect of the proton motive force on riboflavin uptake in whole cells of *L. lactis* NZ9000. Shown is the uptake of radiolabeled riboflavin and alanine (inset) in cells deenergized with 2-deoxyglucose (inverted triangles) and in deenergized cells in which an artificial proton motive force was applied (circles). The expected level of radiolabeled riboflavin inside the cells when no accumulation would take place (the concentration inside equals the concentration in the uptake buffer) is indicated by the dashed line. (B) Chase of internalized radiolabeled riboflavin from NZ9000 cells with unlabeled riboflavin. Uptake of [^{3}H]riboflavin was performed as for Fig. 6 (black circles). After 30 min, unlabeled riboflavin at a final concentration of 70 μM was added, and at the given time points the samples were filtered and radioactivity was counted (white circles).

ATPases, etc.), it is unlikely that ATP directly provides the driving force for transport. Moreover, the reversibility of the transport reaction and the exchange transport both point towards a facilitated diffusion mechanism.

DISCUSSION

The current work is to the best of our knowledge the first molecular and functional analysis of a protein responsible for the transport of riboflavin into a bacterial cell. Vitamin transporters in bacteria appear to fall into a number of different classes of membrane transport proteins. The thiamine (14), vitamin B_{12} (24), and possibly biotin (34) transporters belong to the ABC superfamily, whereas vitamin C transport is mediated by a phosphotransferase system (42), while the Na^+/pantothenate symporter in *E. coli* (40) is an example of secondary transport of a vitamin. Bacteria thus employ different transport mechanisms to internalize various vitamins from their environment. In humans riboflavin is absorbed in the small intestine via a specialized, Na^+-independent carrier-mediated system (35), and in the yeast *Saccharomyces cerevisiae* a riboflavin transporter belonging to a family of monocarboxylate transporters was found that mediates facilitated diffusion (33). We have found that the lactococcal protein RibU most likely mediates riboflavin translocation via a facilitated diffusion mechanism.

Transport assays using radiolabeled riboflavin showed that a deletion in *ribU* renders *L. lactis* incapable of transporting the vitamin. This result was confirmed in the riboflavin-overproducing strain CB010, in which *ribU* expression was essentially undetectable and which consequently exhibited very low riboflavin uptake (not shown). Homologues of RibU are present in gram-positive bacteria and archaea, but homologues were not found in the analyzed genomes of gram-negative bacteria (data not shown). Notably, *E. coli* does not contain a RibU homologue, consistent with its inability to transport riboflavin. Various lactic acid bacteria have also been shown to contain a riboflavin biosynthesis operon (4). The presence of a tightly regulated, dedicated transport system as well as a functional biosynthetic process indicates that such organisms can be flexible and economical in the acquisition of this vitamin depending on environmental supply. However, some species such as *Streptococcus thermophilus* and *Oenococcus oeni* do not appear to possess a riboflavin biosynthetic pathway (4) and therefore must completely depend on a riboflavin uptake system. Members of the RibU family are well conserved, have a length of approximately 200 residues, and are predicted to have five hydrophobic membrane-spanning regions. No homologues of RibU were found in the Transport Classification Database (5), indicating that the family of RibU proteins constitutes a novel addition to the large number of recognized transporter families in the database.

An RFN element is conserved upstream of the *ribU* genes in the analyzed genomes, but the length of this regulatory region varies between species and has different predicted folding conformations. In *B. subtilis* the predicted FMN-responsive element upstream of the *ribU* homologue *ypaA* encompasses 349 nucleotides (41), whereas in *L. lactis* NZ9000 this region is just 246 nucleotides in length as was shown by our transcriptional analysis. Northern hybridization and *lacZ* fusions indicated that transcriptional downregulation occurs in the presence of riboflavin. This would suggest that when riboflavin is present the gene is expressed at a low level but that under riboflavin starvation conditions it increases expression in order to import the vitamin. This is most likely mediated by alternative folding conformations of the 5′ leader region as has been described for

the *rib* operon in *B. subtilis* (41) and *L. lactis* (4), that is, in the presence of FMN or riboflavin the formation of a terminator structure upstream of the *rib* operon is energetically favorable, thus resulting in premature transcription termination. However, in *B. subtilis* it has been suggested that regulation of YpaA expression would occur at the level of translation by means of a Shine-Dalgarno sequence sequestration mechanism (41). In *L. lactis* it is apparent that transcriptional regulation is the dominant means of controlling the expression of the riboflavin transporter, although this does not rule out the possibility that translational regulation may add a second level of control to RibU expression.

The observation that in the *ribU* deletion strain the effect of riboflavin or FMN on regulation of the *rib* operon promoter $P_{ribGBAH}$ is absent suggests that the transporter facilitates uptake of both FMN and riboflavin. Indeed excess FMN competes with uptake of radiolabeled riboflavin. This is in contrast to the situation in *B. subtilis*, where it was reported that FMN is not a substrate of the riboflavin transport system (6). The presence of a high concentration of riboflavin (50 μM) resulted in downregulation of $P_{ribGBAH}$ in NZ9000Δ*ribU*, and the effects were similar to those of 5 μM in the wild-type strain. This implies that at these high levels it is possible for the vitamin to permeate the membrane by some other manner, possibly by diffusion directly through the lipid bilayer or by another, promiscuous transport protein. This is analogous to the situation in *E. coli*, which does not appear to have a dedicated riboflavin transport system. It should be noted, however, that *E. coli* riboflavin auxotrophs require even higher concentrations of riboflavin (720 μM) in their growth medium (2).

It has been shown that disruption of the *ribU* homologue *ypaA* in a *B. subtilis* strain leads to roseoflavin resistance at up to concentrations of 3 mM in contrast to the wild-type strain, which is inhibited at 250 μM to 375 μM (17). *E. coli*, which naturally lacks a riboflavin transport system, has also been found to be resistant to 250 μM roseoflavin, a level which is toxic to *L. lactis* NZ9000. Even though roseoflavin competes with riboflavin uptake by RibU and is likely transported by RibU, NZ9000Δ*ribU* is not resistant to roseoflavin and is even sensitive to concentrations as low as 12 μM (no growth observed; unpublished data). These results suggest that *L. lactis* is more permeable to roseoflavin than either *E. coli* or *B. subtilis*, and it appears that in NZ9000Δ*ribU* the compound enters the cell by some other, as yet unknown means. We found no indication that the proton or sodium motive force plays a role in riboflavin transport. Most likely, RibU mediates facilitated diffusion of riboflavin and equilibration of internal and external riboflavin pools drives the apparent accumulation observed in our experiments. In growing cells, the ATP-dependent conversion of riboflavin into FMN and subsequently into flavin adenine dinucleotide will keep the internal substrate concentration low and ensure net influx of riboflavin. In this way ATP may be indirectly involved in transport.

In conclusion, RibU belongs to a novel protein family involved in riboflavin transport. It is likely a secondary transporter mediating facilitated diffusion of riboflavin and the related compounds roseoflavin and to a lesser extent FMN. Further work is required to establish whether RibU catalyzes uptake alone or in conjunction with other proteins.

ACKNOWLEDGMENTS

This work has been funded by the European Union project QLK1-CT-2000-01376 and an Enterprise Ireland Collaborative Research travel grant IC/2004/095.

We also acknowledge the members of the MG1363 Sequencing Project for the accession to relevant sequence data. D.J.S. acknowledges support from The Netherlands Organization for Scientific Research (NWO) and the Human Frontier Science Program Organization (HFSPO). E.R.G. acknowledges the EU-FP6 program (E-Mep; 504601) for financial support.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Bacher, A., S. Eberhardt, M. Fischer, K. Kis, and G. Richter.** 2000. Biosynthesis of vitamin B2 (riboflavin). Annu. Rev. Nutr. **20:**153–167.
3. **Bacher, A., S. Eberhardt, and G. Richter.** 1996. Biosynthesis of riboflavin, p. 657–664. *In* F. C. Neidhardt, R. Curtis III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
4. **Burgess, C., M. O'Connell-Motherway, W. Sybesma, J. Hugenholtz, and D. van Sinderen.** 2004. Riboflavin production in *Lactococcus lactis*: potential for in situ production of vitamin-enriched foods? Appl. Environ. Microbiol. **70:**5769–5777.
5. **Busch, W., and M. H. Saier, Jr.** 2004. The IUBMB-endorsed transporter classification system. Mol. Biotechnol. **27:**253–262.
6. **Cecchini, G., M. Perl, J. Lipsick, T. P. Singer, and E. B. Kearney.** 1979. Transport and binding of riboflavin by *Bacillus subtilis*. J. Biol. Chem. **254:** 7295–7301.
7. **Cserzo, M., E. Wallin, I. Simon, G. von Heijne, and A. Elofsson.** 1997. Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: the dense alignment surface method. Protein Eng. **10:**673–676.
8. **de Vos, W., and G. Simons.** 1994. Gene cloning and expression systems in lactococci, p. 52–105. *In* M. Gasson and W. de Vos (ed.), Genetics and biotechnology of lactic acid bacteria. Chapman and Hall, Oxford, United Kingdom.
9. **de Vos, W. M., P. Vos, H. de Haard, and I. Boerrigter.** 1989. Cloning and expression of the *Lactococcus lactis* subsp. *cremoris* SK11 gene encoding an extracellular serine proteinase. Gene **85:**169–176.
10. **Fassbinder, F., M. Kist, and S. Bereswill.** 2000. Structural and functional analysis of the riboflavin synthesis genes encoding GTP cyclohydrolase II (*ribA*), DHBP synthase (*ribBA*), riboflavin synthase (*ribC*), and riboflavin deaminase/reductase (*ribD*) from *Helicobacter pylori* strain P1. FEMS Microbiol. Lett. **191:**191–197.
11. **Gelfand, M. S., A. A. Mironov, J. Jomantas, Y. I. Kozlov, and D. A. Perumov.** 1999. A conserved RNA structure element involved in the regulation of bacterial riboflavin synthesis genes. Trends Genet. **15:**439–442.
12. **Griffiths-Jones, S., A. Bateman, M. Marshall, A. Khanna, and S. R. Eddy.** 2003. Rfam: an RNA family database. Nucleic Acids Res. **31:**439–441.
13. **Hofman, K., and W. Stoffel.** 1993. TMbase: a database of membrane spanning protein segments. Biol. Chem. Hoppe-Seyler **347:**166.
14. **Hollenbach, A. D., K. A. Dickson, and M. W. Washabaugh.** 2002. Thiamine transport in *Escherichia coli*: the mechanism of inhibition by the sulfhydryl-specific modifier *N*-ethylmaleimide. Biochim. Biophys. Acta **1564:**421–428.
15. **Horton, R. M., S. N. Ho, J. K. Pullen, H. D. Hunt, Z. Cai, and L. R. Pease.** 1993. Gene splicing by overlap extension. Methods Enzymol. **217:**270–279.
16. **Israelsen, H., S. M. Madsen, A. Vrang, E. B. Hansen, and E. Johansen.** 1995. Cloning and partial characterization of regulated promoters from *Lactococcus lactis* Tn*917-lacZ* integrants with the new promoter probe vector, pAK80. Appl. Environ. Microbiol. **61:**2540–2547.
17. **Kreneva, R. A., M. S. Gelfand, A. A. Mironov, Y. A. Yomantus, Y. I. Kozlov, A. S. Mironov, and D. A. Perumov.** 2000. Inactivation of the *ypaA* gene in *Bacillus subtilis*; analysis of the resulting phenotypic expression. Russ. J. Genet. **36:**972–974.
18. **Krogh, A., B. Larsson, G. von Heijne, and E. L. Sonnhammer.** 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. **305:**567–580.
19. **Kuipers, O., P. G. de Ruyter, M. Kleerebezem, and W. de Vos.** 1998. Quorum sensing-controlled gene expression in lactic acid bacteria. J. Biotechnol. **64:**15–21.
20. **Kuipers, O. P., M. M. Beerthuyzen, R. J. Siezen, and W. M. De Vos.** 1993. Characterization of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis*. Requirement of expression of the *nisA* and *nisI* genes for development of immunity. Eur. J. Biochem. **216:**281–291.
21. **Leenhouts, K., G. Buist, A. Bolhuis, A. ten Berge, J. Kiel, I. Mierau, M. Dabrowska, G. Venema, and J. Kok.** 1996. A general system for generating unlabelled gene replacements in bacterial chromosomes. Mol. Gen. Genet. **253:**217–224.

22. **Leenhouts, K. J., J. Kok, and G. Venema.** 1989. Campbell-like integration of heterologous plasmid DNA into the chromosome of *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. **55:**394–400.
23. **Leenhouts, K. J., J. Kok, and G. Venema.** 1991. Replacement recombination in *Lactococcus lactis*. J. Bacteriol. **173:**4794–4798.
24. **Locher, K. P., A. T. Lee, and D. C. Rees.** 2002. The *Escherichia coli* BtuCD structure: a framework for ABC transporter architecture and mechanism. Science **296:**1091–1098.
25. **Mironov, A. S., I. Gusarov, R. Rafikov, L. E. Lopez, K. Shatalin, R. A. Kreneva, D. A. Perumov, and E. Nudler.** 2002. Sensing small molecules by nascent RNA: a mechanism to control transcription in bacteria. Cell **111:** 747–756.
26. **Otto, R., B. ten Brink, H. Veldkamp, and W. N. Konings.** 1983. The relation between growth rate and electrochemical gradient in *Streptococcus cremoris*. FEMS Microbiol. Lett. **16:**69–74.
27. **Perkins, J. B., and J. Pero.** 2002. Vitamin biosynthesis, p. 271–286. *In* A. Sonenshein, J. Hoch, and R. Losick (ed.), *Bacillus subtilis* and its closest relatives: from genes to cells. ASM Press, Washington, D.C.
28. **Poolman, B., and W. N. Konings.** 1988. Relation of growth of *Streptococcus lactis* and *Streptococcus cremoris* to amino acid transport. J. Bacteriol. **170:** 700–707.
29. **Poolman, B., D. Molenaar, and W. N. Konings.** 1994. Diversity of transport mechanisms in bacteria, p. 329–379. *In* M. Shinitzky (ed.), Biomembranes: structural and functional aspects, vol. 2. Balaban Publishers, Rehovot, Israel.
30. **Poolman, B., E. J. Smid, and E. J. Konings.** 1987. Kinetic properties of a phosphate-bond driven glutamate/glutamine transport in *Streptococcus lactis* and *Streptococcus cremoris*. J. Bacteriol. **169:**2755–2761.
31. **Poolman, B., E. J. Smid, H. Veldkamp, and W. N. Konings.** 1987. Bioenergetic consequences of lactose starvation for continuously cultured *Streptococcus cremoris*. J. Bacteriol. **169:**1460–1468.
32. **Pujic, P., R. Dervyn, A. Sorokin, and S. D. Ehrlich.** 1998. The *kdgRKAT* operon of *Bacillus subtilis*: detection of the transcript and regulation by the *kdgR* and *ccpA* genes. Microbiology **144:**3111–3118.
33. **Reihl, P., and J. Stolz.** 2005. The monocarboxylate transporter homologue Mch5p catalyzes riboflavin (vitamin B2) uptake in *Saccharomyces cerevisiae*. J. Biol. Chem. **280:**39809–39817.
34. **Rodionov, D. A., A. A. Mironov, and M. S. Gelfand.** 2002. Conservation of the biotin regulon and the BirA regulatory signal in Eubacteria and Archaea. Genome Res. **12:**1507–1516.
35. **Said, H. M.** 2004. Recent advances in carrier-mediated intestinal absorption of water-soluble vitamins. Annu. Rev. Physiol. **66:**419–446.
36. **Sambrook, J., and D. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
37. **Sybesma, W., C. Burgess, M. Starrenburg, D. van Sinderen, and J. Hugenholtz.** 2004. Multivitamin production in *Lactococcus lactis* using metabolic engineering. Metab. Eng. **6:**109–115.
38. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophage. Appl. Microbiol. **29:**807–813.
39. **Thompson, J., and B. M. Chassy.** 1985. Intracellular phosphorylation of glucose analogs via phosphoenolpyruvate:mannose-phosphotransferase system in *Streptococcus lactis*. J. Bacteriol. **162:**224–234.
40. **Vallari, D. S., and C. O. Rock.** 1985. Pantothenate transport in *Escherichia coli*. J. Bacteriol. **162:**1156–1161.
41. **Winkler, W. C., S. Cohen-Chalamish, and R. R. Breaker.** 2002. An mRNA structure that controls gene expression by binding FMN. Proc. Natl. Acad. Sci. USA **99:**15908–15913.
42. **Zhang, Z., M. Aboulwafa, M. H. Smith, and M. H. Saier, Jr.** 2003. The ascorbate transporter of *Escherichia coli*. J. Bacteriol. **185:**2243–2250.
43. **Zuker, M.** 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. **31:**3406–3415.